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Abstract on next page.

15. SUBJECT TERMS

Subject terms on next page.

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14. ABSTRACT

The purpose of this project is to identify initial biomarker patterns in SLE nephritis using screening proteomic profiling. Subject recruitment has been completed. Utility of one of the biomarkers (NGAL) in predicting worsening of global and renal SLE disease activity has been validated. We have identified novel urinary biomarkers that distinguish between class IV and class V lupus nephritis, including α-1-B glycoprotein by 2D gel electrophoresis, α-1-antitrypsin by SELDI-TOF-MS, citrate and taurine by NMR spectroscopy-based metabolomic profiling, and apolipoprotein D, lipocalin-like prostaglandin D synthetase, hemopexin, ceruloplasmin, α-1-B glycoprotein and orosomucoid by LC-MS/MS profiling. These important findings need validation. First, we need to validate whether these biomarkers are differentially expressed in patients with kidney biopsy-proven lupus nephritis types IV and V. Another important component of validation will be to utilize a different assay to confirm the novel findings on proteomic profiling. These are our goals for the upcoming year. Overall, these studies will identify a subset of non-invasive biomarkers that identify lupus nephritis sub-classes, and predict the clinical course of the disease. The significance of such biomarkers is that they will provide novel non-invasive tools to identify patients with lupus nephritis, to risk-stratify the subjects for therapies, and to follow the efficacy of therapies.

15. SUBJECT TERMS

Lupus nephritis, Biomarkers, Proteomics

Table of Contents

INTRODUCTION

In our original proposal, we proposed to identify biomarker patterns in SLE nephritis by pursuing the following specific aims:

Specific Aim 1: Screening proteomic profiling: Initial high-throughput screening proteomic analysis will be done in the Devarajan Lab using 2D gel electrophoresis and Surface-Enhanced Laser Desorption/ Ionization Time-of-Flight mass spectrometry (SELDI-TOF-MS). Changes in proteomic profiles will be confirmed and enhanced using NMR- and MS-based metabonomics, by Dr. Michael Kennedy, Miami University. Changes in proteomic profiles will be compared to changes in currently available renal biomarkers (urinalysis, blood and urine chemistry), medications and other clinical outcomes (overall disease activity, renal and overall damage).

Specific Aim 2: Advanced proteomic profiling: Advanced proteomic studies on selected sample sets will be performed at Applied Biotechnology Branch, Air Force Research Lab, Wright-Patterson Air Force Base (AFRL/HEPB), where LC/MS based protein profiling using Thermo LTQ FT-ICR will provide ultra-high resolution/mass accuracy protein identification, using the LTQ FT-ICR hybrid instrument (Thermo Electron North America LLC).

Aim 1 has essentially been completed. The utility of one of the biomarkers (NGAL) in predicting worsening of global and renal SLE disease activity has been validated. We have identified novel urinary biomarkers that distinguish between class IV and class V lupus nephritis, including α-1-B glycoprotein by 2D gel electrophoresis, α-1-antitrypsin by SELDI-TOF-MS, citrate, taurine and hippurate by NMR spectroscopy-based metabolomic profiling, and apolipoprotein D, lipocalin-like prostaglandin D synthetase, hemopexin, ceruloplasmin, α-1-B glycoprotein and orosomucoid by LC-MS/MS profiling.

Aim 2 could not be completed by our collaborators at the Wright-Patterson Air Force Base due to unexpected and unanticipated technical difficulties. However, the exciting results from Aim 1 need validation. First, we need to validate whether these biomarkers are differentially expressed in patients with kidney biopsy-proven lupus nephritis types IV and V. Another important component of validation will be to utilize a different assay to confirm the novel findings on proteomic profiling. These are our goals for the upcoming year. We have requested and have been granted a No Cost Extension for an additional year in order to complete these validation studies.

Overall, these studies will identify a subset of non-invasive biomarkers that identify lupus nephritis sub-classes, and predict the clinical course of the disease. The significance of such biomarkers is that they will provide novel non-invasive tools to identify patients with lupus nephritis, to risk-stratify the subjects for therapies, and to follow the efficacy of therapies.

BODY

Research Accomplishments for Task 1: To identify initial biomarker patterns in SLE nephritis using screening proteomic profiling

1.1:

Urinary Metabonomic studies in Lupus Nephritis

Class IV and V Lupus nephritis (LN) show different histological features and differ in prognosis. We aimed to identify non-invasive metabonomic biomarkers which differentiate between class IV and V LN. Metabolic profiling was conducted using urine samples of patients with proliferative LN without membranous features (n=7) and pure membranous LN (n= 7). As disease controls, 10 patients with focal segmental glomerulosclerosis (FSGS) and proteinuria were also examined. Urinary profiling was performed using NMR- and MS-based metabonomics at Miami University, in collaboration with the laboratory of Dr. Michael Kennedy. Information about demographic and clinical data was obtained for each patient (please see Figure 1). Metabolic profiling analysis was done by visual inspection and principal component analysis (please see Figures 2 and 3). Preliminary analysis performed during the last year has revealed that urinary concentrations of the metabolite taurine were significantly elevated ($p < 0.01$) in membranous LN as compared to proliferative LN patients. There was also a trend towards lower urinary concentrations of the metabolite citrate with pure membranous as compared to proliferative LN ($p < 0.1$). In addition, urinary concentrations of the metabolite hippurate were significantly increased ($p < 0.001$) with membranous LN as compared to FSGS but not different from proliferative LN. The pilot results indicate that there are differences in urinary metabolite profiles of proliferative as compared to membranous LN. Urine metabolites may be biomarkers that can discriminate between membranous and proliferative LN. The statistical analysis, including correlations with clinical features, will be completed in the upcoming year, and a manuscript will be submitted for publication.

		Focal segmental glomerulosclerosis		Proliferative LN (class III or IV)		Pure membranous LN (class V)	
		n of 10 (%)	Median (Range)	n of 7 (%)	Median (Range)	n of 7 (%)	Median (Range)
Females		3 (33%)		4/7 (57%)		5/7 (71%)	
Race	Black	2/10 (20%)		3/7 (42%)		3/7 (42%)	
	White	5/10 (50%)		2/7 (29%)		2/7 (29%)	
	Other	3/10 (30%)*		2/7 (29%)†		3/7 (43%) **	
Medications	Oral prednisone	4/10 (40%)		7/7 (100%)		4/7 (57%)	
	Mycophenolate mofetil	5/10 (50%)		3/7 (43%)		5/7 (71%)	
	Cyclophosphamide	-		2/7 (29%)		1/7 (14%)	
	Angiotensin blocking agent	9/10 (90%)		2/7 (29%)		6/7 (86%)	
Kidney Status	GFR < 60 ml/min/m^2	4/10 (40%)		1/7 (14%)		1/7 (14%)	
	Protein: creatinine ratio > 0.5	5/5 (100%)		7/7 (100%)		7/7 (100%)	
	Renal SDI score > 0¥	-		0/3		1/4 (25%)	
	Renal SLEDAI score‡	-			4 (0-16)		8 (4-12)
	Presence of anti double-stranded-dsDNA	-		7/7 (100%)		4/7 (67%)	

‡ Systemic Lupus Erythematosus Disease activity Index (SLEDAI) - renal component score; ¥ Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index - renal component score; *American Indian 1, Asian 1, Mixed racial 1; † Asian 1, Mixed racial 1; ** Asian 1, Unknown

Figure 1. Patient demographics, medications, and renal status at the time of urine collection

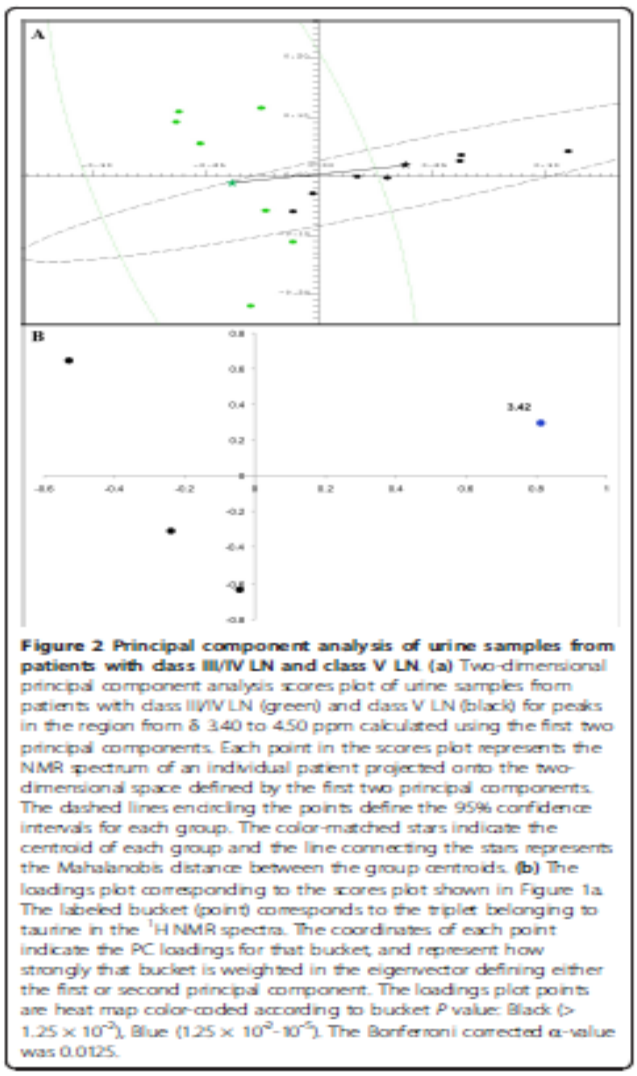

Figure 2 Principal component analysis of urine samples from patients with class III/IV LN and class V LN. (a) Two-dimensional principal component analysis scores plot of urine samples from patients with class III/IV LN (green) and class V LN (black) for peaks in the region from δ 3.40 to 4.50 ppm calculated using the first two principal components. Each point in the scores plot represents the NMR spectrum of an individual patient projected onto the two-dimensional space defined by the first two principal components. The dashed lines encircling the points define the 95% confidence intervals for each group. The color-matched stars indicate the centroid of each group and the line connecting the stars represents the Mahalanobis distance between the group centroids. **(b)** The loadings plot corresponding to the scores plot shown in Figure 1a. The labeled bucket (point) corresponds to the triplet belonging to taurine in the ^{1}H NMR spectra. The coordinates of each point indicate the PC loadings for that bucket, and represent how strongly that bucket is weighted in the eigenvector defining either the first or second principal component. The loadings plot points are heat map color-coded according to bucket *P* value: Black (> 1.25×10^{-2}), Blue (1.25×10^{-2}-10^{-5}). The Bonferroni corrected α-value was 0.0125.

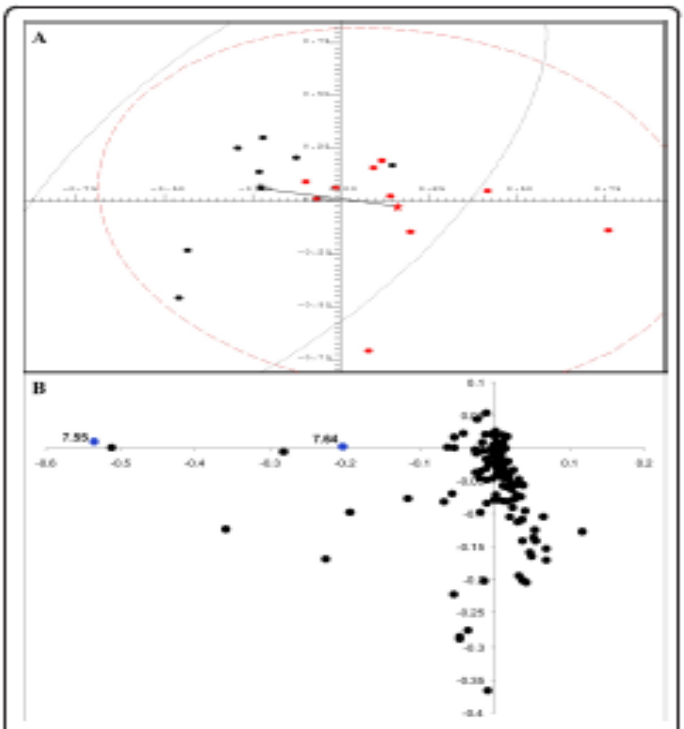

Figure 3 Principal component analysis of urine samples from class V LN patients and focal segmental glomerulosclerosis patients. (a) Two-dimensional principal component analysis scores plot of urine samples from patients with class V LN patients (black) and focal segmental glomerulosclerosis patients (red) using the first two principal components. The dashed lines encircling the points define the 95% confidence intervals for each group. The color-matched stars indicate the centroid of each group and the line connecting the stars represents the Mahalanobis distance between the group centroids. **(b)** The loadings plot corresponding to the scores plot in Figure 2a. The buckets shown are in the region from δ 0.02 to 10.0 ppm. The loadings plot is heat map color-coded according to bucket *P* values: Black (> 1.730×10^{-4}), Blue 1.730×10^{-4}-10^{-5}). The Bonferroni corrected α-value was 1.730×10^{-4}.

Research Accomplishments for Task 2: To identify biomarkers predictive of SLE nephritis using advanced proteomic profiling

2.1: Advanced Proteomics in Lupus Nephritis

These studies were initiated at the Applied Biotechnology Branch, Air Force Research Lab, Wright-Patterson Air Force Base (AFRL/HEPB), under the direction of Dr. Schlager. LC/MS based protein profiling of urine from SLE patients using Thermo LTQ FT-ICR was expected to provide ultra-high resolution/mass accuracy protein identification. However, these studies could not be completed by our collaborators at the Wright-Patterson Air Force Base due to unexpected and unanticipated technical difficulties.

However, our collaborators were able to complete preliminary LC-MS/MS2 studies, which have uncovered several differences between the groups. Among proteins upregulated in Class V LN were apolipoprotein D, lipocalin-like prostaglandin D synthetase, ITIH4, Caspase 10, uromodulin and

CD14. Those most upregulated in Class IV LN were vitamin D binding protein, ceruloplasmin, hemopexin, A1BG and orosomucoid. A1AT has been linked to SLE flares and hemopexin is associated with glomerular disease. It will be important in the upcoming year to confirm these preliminary findings using additional samples from patients with Class IV and Class V lupus nephritis.

Overall, these studies will identify a subset of non-invasive biomarkers that identify lupus nephritis sub-classes, and predict the clinical course of the disease. The significance of such biomarkers is that they will provide novel non-invasive tools to identify patients with lupus nephritis, to risk-stratify the subjects for therapies, and to follow the efficacy of therapies.

KEY RESEARCH ACCOMPLISHMENTS DURING MOST RECENT YEAR

- Identification of urinary biomarkers that distinguish between class IV and class V LN, that includes the following differentially expressed biomarkers:
    - Citrate, taurine and hippurate by NMR spectroscopy-based metabolomic profiling
    - apolipoprotein D, lipocalin-like prostaglandin D synthetase, hemopexin, ceruloplasmin, α-1-B glycoprotein and orosomucoid by LC-MS/MS profiling

REPORTABLE OUTCOMES

ABSTRACT PRESENTED:

- Michael R. Bennett, PhD, Michiko Suzuki, MD, PhD, Shannen Nelson, Josh Pendl, Michael Kennedy, Pavel Shyianov, and Hermine Brunner, MD, Prasad Devarajan, MD. Urinary biomarkers to distinguish Class IV vs Class V lupus nephritis. Abstract presented at the Annual Meeting of the American Society of Nephrology, 2010, and the American College of Rheumatology Annual Meeting, 2010

MANUSCRIPT IN PREPARATION:

- Lindsey Romick-Rosendale, Hermine Brunner, Michael Bennett, Rina Mina, Shannen Nelson, Prasad Devarajan, and Michael A. Kennedy. Identification Of Candidate Urinary Biomarkers To Distinguish Proliferative From Membranous Lupus Nephritis by NMR-Based Metabonomics

CONCLUSION

Thus far, we have completed Task 1 and a portion of Task 2. We have completed subject recruitment, validated one of the biomarkers (NGAL) as a predictive urinary biomarker for impending worsening of SLE disease activity, and identified a urinary biomarker signature that distinguish between class IV and class V LN. This includes albumin fragments (25kDa) and α-1-B glycoprotein (60kDa) identified by 2D gel electrophoresis, α-1-antitrypsin by SELDI-TOF-MS, citrate, taurine and hippurate by NMR spectroscopy-based metabolomic profiling, and apolipoprotein D, lipocalin-like prostaglandin D synthetase, hemopexin, ceruloplasmin, α-1-B glycoprotein and orosomucoid by LC-MS/MS profiling.

Additional proteomic profiling studies using LC/MS based protein profiling using Thermo LTQ FT-ICR were initiated, but could not be completed during the past year, due to several technical and equipment-related difficulties encountered by the Wright Patterson Air Force Base laboratories.

Overall, these studies will identify a subset of non-invasive biomarkers that identify lupus nephritis sub-classes, and predict the clinical course of the disease. The significance of such biomarkers is that they will provide novel non-invasive tools to identify patients with lupus nephritis, to risk-stratify the subjects for therapies, and to follow the efficacy of therapies.

Research Accomplishments planned for the Upcoming Year

The exciting results from Aim 1 and Aim 2 completed thus far need validation. First, we need to validate whether these biomarkers are differentially expressed in patients with kidney biopsy-proven lupus nephritis types IV and V. We will need to correlate changes in kidney biopsy with changes in non-invasive urinary biomarker excretion patterns. Another important component of validation will be to utilize a different assay to confirm the novel findings on proteomic profiling. These are our goals for the upcoming year. We have requested and have been granted a No Cost Extension for an additional year in order to complete these validation studies. Our specific aims for the upcoming year are as follows:

Upcoming Year Aim 1:

First, we need to validate whether these biomarkers are differentially expressed in patients with kidney biopsy-proven lupus nephritis types IV and V. We also need to correlate the differential expression of the biomarkers with structural and histologic changes seen on kidney biopsies. We have information on results of kidney biopsies from a number of recruited patients in whom we also have urine samples obtained very close to the biopsy dates. Since the measurement of several of the biomarkers has already been completed, what remains to be done is (a) analysis of biopsy results to identify specific features of lupus activity and lupus chronicity, (b) correlation of measured biomarkers with the identified histologic features of lupus activity and lupus chronicity, and (c) comparison of associations between histology and novel biomarkers versus traditional biomarkers (such as urine protein, serum complement protein C3, and estimated glomerular filtration rate). These studies will establish the relationship of the novel urinary biomarkers as well as the established measures of renal function to the histological findings with lupus nephritis, and will identify certain combinations of the biomarkers that are diagnostic of specific histological features with lupus nephritis. The identified association of novel as well as traditional biomarkers of lupus nephritis with

specific histological features bears the expectation that longitudinal non-invasive measurement of lupus nephritis will become feasible, and will allow for a more effective and personalized monitoring of lupus nephritis and its therapy.

Upcoming Year Aim 2:

Another important component of validation will be to utilize a different assay to confirm the novel findings on proteomic profiling. The differential expression of α-1-B glycoprotein discovered by 2D gel electrophoresis will be validated by ELISA and by Western Blots. The differential expression of α-1-antitrypsin identified by SELDI-TOF-MS will be validated by Western Blot. The differential expression of citrate, taurine and hippurate identified by metabolic profiling will be validated by direct laboratory platform measurements. If validated, this set of studies will provide additional validated biomarkers to be used for non-invasive monitoring of lupus nephritis.

The two aims are a natural extension of our findings thus far. Completion of these two additional aims will be tremendously value added to the studies completed thus far, and will very likely result in two additional manuscript publications.

Although the work has progressed very well, we must remain cognizant of potential problems in the future, and we must be prepared to address these problems, as summarized below:

(a) Current problems that may impede performance

- Initial SELDI-TOF-MS studies have revealed more than 30 proteins that are differentially expressed in Class IV versus Class V lupus nephritis. This number is larger than initially anticipated. Thus far, we have been able to identify and validate only one of these candidates, namely α-1-antitrypsin. We will need to perform additional SELDI-TOF-MS experiments on additional samples, in order to establish whether the observed large differences are consistent in a new set of samples.
- Initial 2 dimensional gel electrophoresis (2DGE) and SELDI-TOF-MS have identified only 2 proteins significantly over-expressed in class IV vs. class V: human serum albumin fragments (25kDa) and α-1-B glycoprotein (60kDa). This number is smaller than initially anticipated.
- Initial NMR spectroscopy-based metabolomic profiling have identified only 2 species differentially expressed in class IV vs. class V: citrate and taurine. This number is smaller than initially anticipated.

(b) Anticipated problems

- Initial LC/MS based protein profiling of urine from SLE patients using Thermo LTQ FT-ICR has proven to be more expensive and associated with more technical difficulties than initially anticipated. These experiments will not be completed. Instead, we have proposed important validation studies for the upcoming year, as mentioned above.